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14. ABSTRACT
Inappropriate aggregation of peptides and proteins is a major concern for biotechnology and is also associated with several major human pathological conditions. The goal of this project is to develop a robust method for refolding proteins and peptides by adapting novel protein salvage pathways from extremophiles to operate under mild conditions. In prior studies, we have shown that the chaperones from extremophiles are exceptionally stable and versatile, reflecting their role in folding and salvaging proteins under extreme conditions. The remarkably thermostable small Heat Shock Protein from the psychrotolerant archaeon Methanococcoides burtonii is our first choice for a "holdase" chaperone. This crystallin-like chaperone is stable from 15-55°C, and maintains aggregation-prone target proteins in the soluble state, without denaturants. In addition to applications of this pathway to recombinant proteins in solution and during expression in vivo, we propose to immobilize the chaperones to form a chaperone matrix

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Extremophiles, chaperones, protein folding, psychrophile

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(YIP 07) INFLUENCE OF TEMPERATURE ON THE DYNAMIC STRUCTURES OF PSYCHROPHILIC SMALL HEAT SHOCK PROTEINS

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Program goals

1. To identify the chaperone functions and co-chaperone partners of the small heat shock protein (sHsp) from a psychrophilic archaeon, *M. burtonii* (Mb-sHsp).

2. To obtain the solution and crystal structures of the *M. burtonii* small heat shock.

3. To determine the functions of the *M. burtonii* small heat shock proteins

Our interest in the psychrophile chaperones stems from the assumption that production of correctly folded proteins at or below 4^0C represents a significant barrier that, it overcome will be of utility in maintaining proteins during freeze/thaw stress and under cold denaturation. Previous results have established that the *Methanococcoides burtonii* small Heat Shock protein has an extraordinary range of operating temperatures and its binding to the chaperonin in a coupled folding reaction has been described (Laksanalamai et al, 2008). In this work in Year 1 and 2 of the project we a followed up on the initial experiment shown in Figure 1, in which an active, wild type Small Heat Shock Protein was covalently amide-linked to a Biacore chip and its capacity to adsorb specifically to non-native lysozyme was established. Binding to native lysozyme was negligible. Knowing that the sHSP does not carry out folding but has a remarkable capacity for maintaining non-native proteins in solution. The major question we addressed was, where do the non-native proteins go next, on their progress towards refolding? This question has not been answered although an earlier paper of ours (Laksanalamai et al, 2006) established that a cooperative salvage pathway could be observed using taq DNA polymerase with the chaperones from the hyperthermophile Pyrococcus furiosus. Methods developed in this study were modified and applied to the sHSP and chaperonin from the psychrophile Methanococcoides burtonii (Laksanalamai et al, 2009).

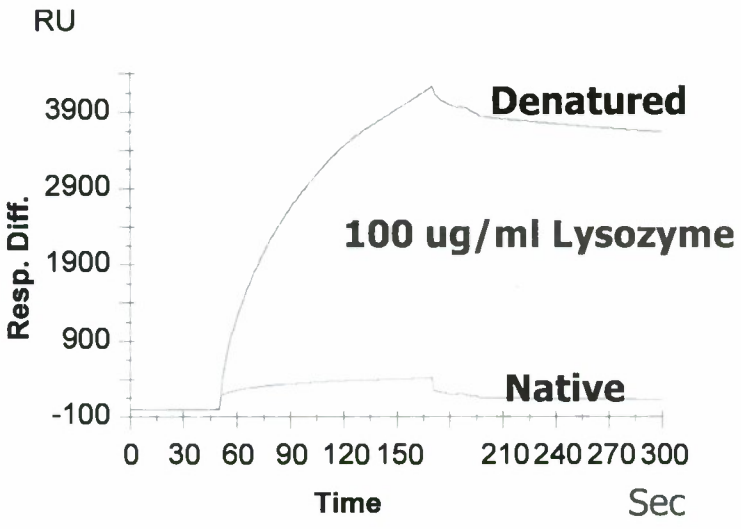

Figure 1. Demonstration of specific adsorption of non-native targets by small heat shock protein using surface plasmon resonance signals in the Biacore T100.

We developed an assay for folding efficiency of archaeal chaperones in vivo shown in Figure 2. We coexpressed seven different chaperones with Green Fluorescent Protein individually, taking advantage of the known slow-folding property of GFP. If the recombinant chaperones are effective in E. coli, as is the case for lanes 2, 5, 6 and 8 representing *P. furiosus* HSP60, *P. furiosus* nascent Associated Complex, *M. burtonii* HSP60, and *M. jannaschii* g PFD, then

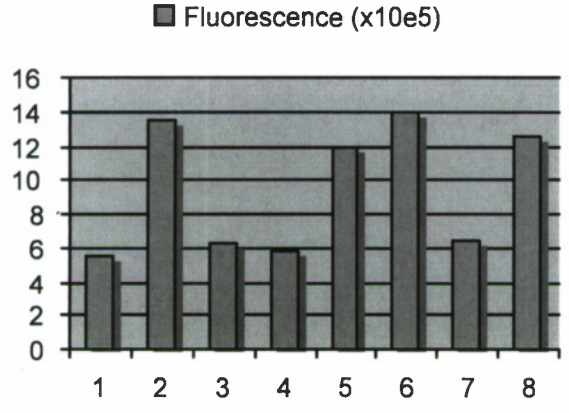

Figure 2. Promotion of GFP fluorescence by chaperones. Fluorescence signal intensity of GFP is indicated in arbitrary units. Shown are whole cell measurements two hours after co-induction of GFP plus the indicated chaperone. 1, control lacking chaperone; 2, *P. furiosus* HSP60; 3, *P. furiosus* α PFD; 4, *P. furiosus* β PFD; 5, *P. furiosus* NAC; 6, *M. burtonii* HSP60; 7, *M. burtonii* sHSP; 8, *M. jannaschii* γ PFD.

the fluorescence of the cells is enhanced. Notably, *M. burtonii* sHSP by itself does not enhance GFP folding, whereas the recombinant Mb HSP60 does. Our hypothesis was that the in vivo function for sHSP involves an exchange of non-native folding substrates from the sHSP to the HSP60. The latter is the only ATP dependent folding nanomachine predicted from the genome annotation of *M. burtonii*.

On the other hand, the sHSP proved to have exceptional dispersive properties for refractory protein aggregates.

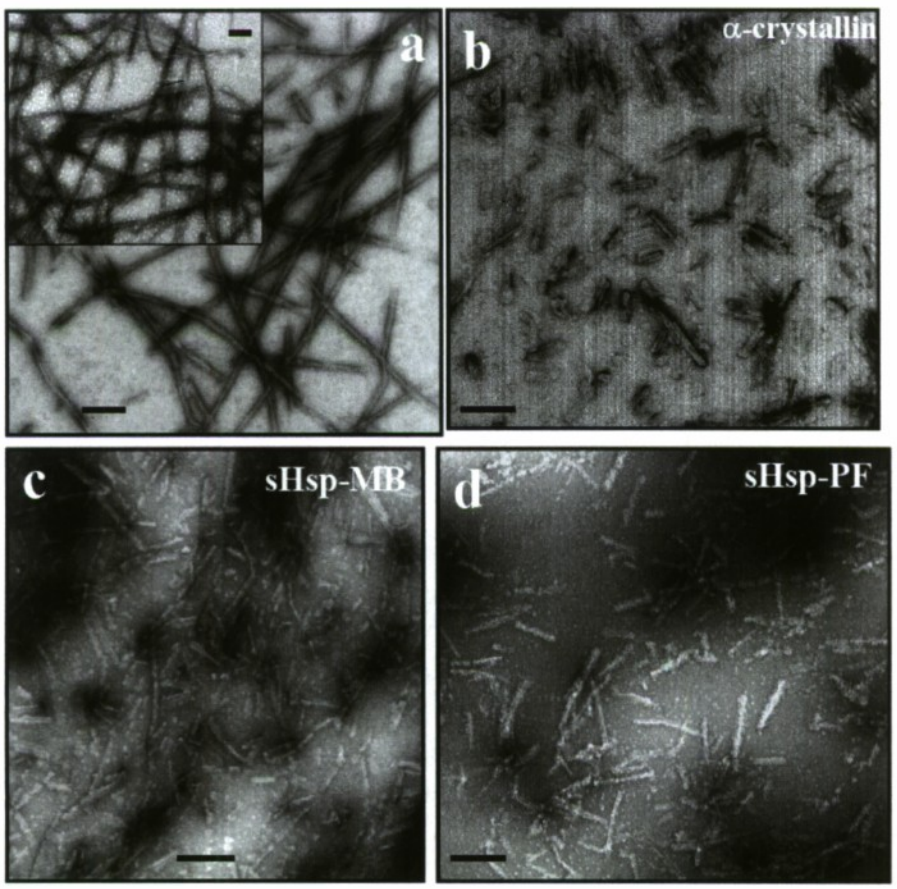

Figure 3. Electron microscopy imaging of amyloid fibrils incubated with sHsps. Amyloid fibrils incubated for 1 hour at 37 °C in the absence of sHsps (*a*), or in the presence of α-crystallin (*b*), sHsp-MB (*c*), or sHsp-PF (*d*). Fibrils were formed in 2 M GdnHCl at 37 °C and dialyzed out of GdnHCl prior to incubation with sHsps. The molar ratio of rPrP to specific sHsp was 20:1. Scale bars = 0.2 um.

We adapted the Biacore experiment shown in Figure 1 to test the hypothesis that a handoff occurs between sHSP and HSP60. As shown in Figure 3, we loaded a sHSP-chip with denatured lysozyme and then tested the capacity

of HSP60 to offload the bound lysozyme. The offloading trajectory was unaltered in the experiments with ATP and buffer alone (controls), but was accelerated by the addition of Mb-Cpn and significantly activated by the addition of ATP.

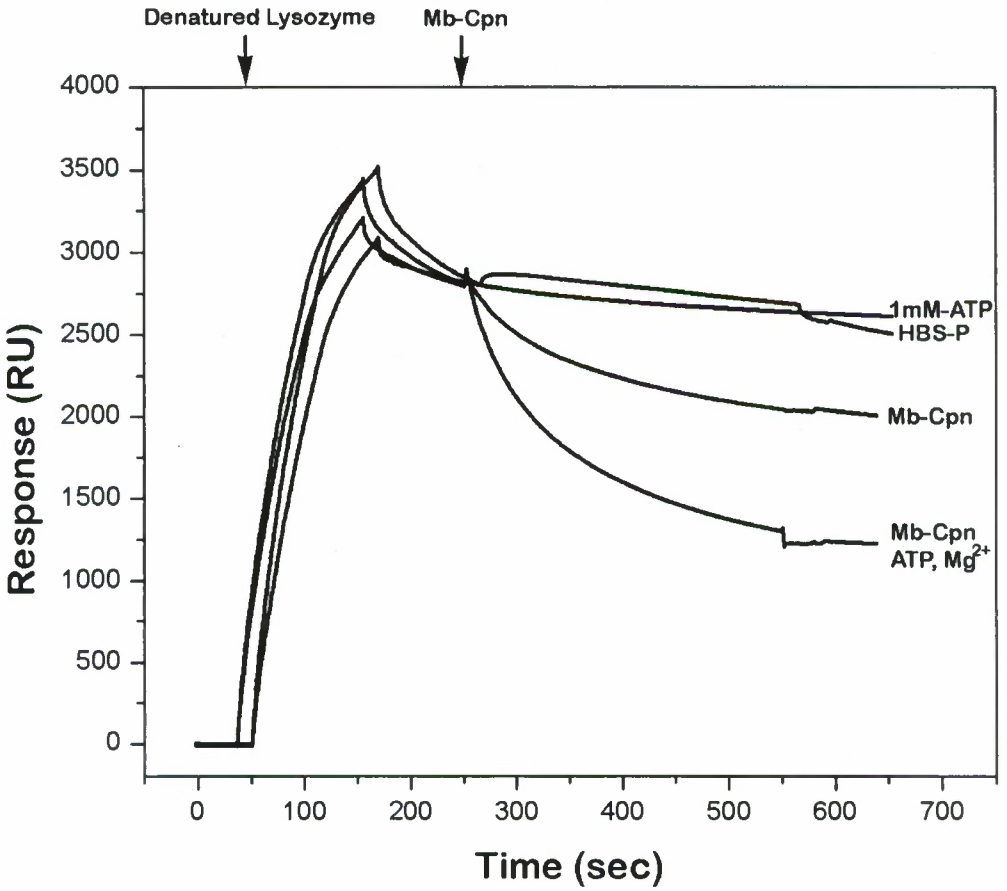

Figure 4. Surface plasmon resonance spectroscopy of the offloading of heat-denatured lysozyme from Mb-sHsp to Mb-Cpn. 100μg/ml of heat-denatured lysozyme was injected on a sensor chip that has immobilized Mb-sHsp. 30 μg/ml of Mb-Cpn was injected to offload bound denatured lysozyme from Mb-sHsp as indicated.

Accomplishments (Years 1 and 2):

- **Established interchaperone exchange of denatured lysozyme between Mb-sHSP and Mb-HSP60 (Laksanalamai et al, (2008)**
- **Determined minimal complex (24-mer) of Mb-sHSP**
- **Dissociated highly refractory prion amyloid fibrils under mild conditions using Mb-Shsp (Sun et al, (2008) J. Molec. Biol 376, 1155-1164)**
- **Patent submitted - recombinant protein refolding**

Publications/Patents
September 2007- December 2009

Laksanalamai, P, S. Narayan, H. Luo and F. T. Robb (2008) Chaperone action of a versatile small heat shock protein from *Methanococcoides burtonii*. A cold-adapted archaeon. Proteins

Sun, Y, N Makarava, C-I Lee, **P Laksanalamai,** FT Robb, IV Baskakov (2008) Conformational Stability of PrP Amyloid Fibrils Controls Their Smallest Possible Fragment Size Journal of Molecular Biology 376 (4) 1155-1167.

Robb, FT and **P. Laksanalamai.** 2008. Thermophilic Protein-Folding Systems pp 55-71 in Thermophiles: Biology and Technology at High Temperatures eds: Frank Robb, Garabed Antranikian, Dennis Grogan, Arnold Driessen ISBN 0849392144

Robb, FT and H. Smith (2007) PCT filed **Chaperone assisted protein folding**

Results from Year 3: Dissection of sHSP functions by complementation and mutational analysis.

1. **Enzyme salvage and refolding experiments.**
We used bovine glutamate dehydrogenase (a labile hexamer with Mw of 336 kDa) in a demonstration of the ability of sHSP to stabilize for long time periods at supra0optimal temperatures

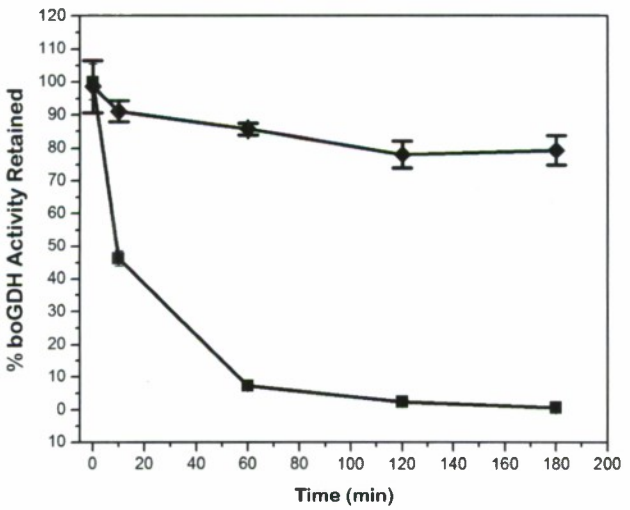

Figure 5. Protection of bovine glutamate dehydrogenase (boGDH) by Mb-sHsp. Bovine GDH activity was retained after heating with Mb-sHsp (◆) and without Mb-sHsp (■) Bovine GDH was heated at 42°C for the indicated time,

As shown in Figure 5, we tested the Mb-sHSP for chaperone activity at 42^{0}C and showed that it could **maintain the GDH activity at 80%.for three hours while the activity in the control was dead after one hour.**

We then explored the possibility that combinations of sHSPs could be used in the salvage bovine glutamate dehydrogenase. As shown in Figure 6, we tested the Mb-sHSP in combination with two sHSP paralogs from the extreme thermophile *Carboxydothermus hydrogenoformans*. The aim of these experiments was to determine whether the sHSPs would synergize in their activity at an intermediate temperature bridging the optima for their source organisms. *M. burtonii* has an optimal growth in the range from 18-23^{0}C whereas *C. hydrogenoformans* grows optimally at 72^{0}C. Three different Ch-sHSPs were tested in combination with Mb-sHSP.

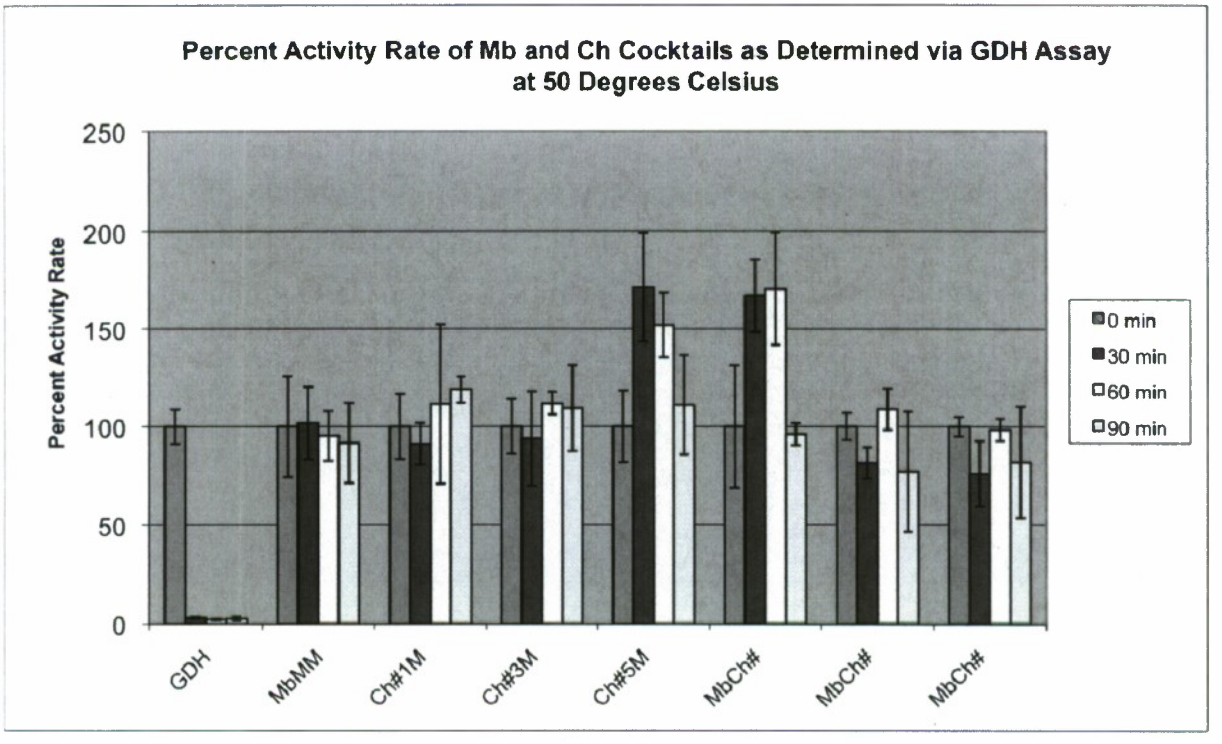

Figure 5. GDH salvage reaction with combined *M. burtonii* and *C. hydrogenoformans* sHSPs. Bovine GDH was stabilized and, in experiments 5 and 6, refolded above initial activity.
Experiments from left: 1. GDH alone, 2. GDH with Mb sHSP in EPPS buffer, 3. GDH with Ch sHSP#1, 4. GDH with Ch sHSP#3, 5. GDH with Ch sHSP #5, 6. MbCh#1 combined sHSPs, 7. MbCh#3 combined sHSPs, 8. MbCh#5 combined sHSPs..

As shown in Figure 5, bovine GDH denatured rapidly at 50^{0}C, with activity declining to 10% of the initial value in 30 minutes. The individual sHSPs had stabilizing effects on boGDH such that it's temperature range for 100% activity was extended to 60 minutes at 50^{0}C. Ch sHSP#5 by itself had a very significant

refolding reaction, which was also observed in the combination of Mb-sHSP and Ch-sHSP#1 (experiment 6).

In both cases where the GDH activity of this preparation as supplied by Sigma-Aldrich was increased in activity to 170% of the initial value, we interpret this as evidence that the enzyme as supplied was partially denatured and was salvaged by chaperone action. .

Mutational analysis of Mb sHSP.

In order to identify the functional domains of the Mb-sHSP, we undertook a mutaional analysis of the gene and measured the chaperone activities of the mutant chaperones using bovine GDH as a target protein to measure residual chaperone activity.

The Mb-sHSP mutants E73K, P104A, CΔ8 CΔ9 and NΔ20 were expressed and found to be defective in boGDH stabilization in the rank listed. Surprisingly, the double deletion in both C and N termini with a molecular weight of 14 kDa is an effective and thermostable minichaperone. The N-terminal deletion alone has chaperone activity similar to the wild type, which raises questions about the functions of the N-terminus. However, stabilization of E. coli to lethal heat shock, a measure of in vivo activity, is dead in all mutants except the P140A mutant. The internal loop deletion mutant, that is unable to form dimers, predictably has the poorest chaperone activity. Chaperone activities and mutation types are summarized below in Table 1.

Mutant	Mutation Strategies	Oligomer status	Protectio of GDH	*E.coli* survival
WT	None	**24-mer**	**++++**	**++++**
C8	C-terminal deletion	**24-mer**	**+++**	**-**
C9	C-terminal deletion	**2-mer**	**++**	**-**
dNC8	C and N-terminal deletions	**24-mer**	**++**	**-**
dNC9	C and N-terminal deletio	**2-mer**	**++**	**-**
dN	N-terminal deletion	**24-mer**	**++++**	**-**
P140A	Point mutation	**24-mer**	**++**	**++++**
E73K	Point mutation	**N-mer /filament**	**+**	**ND**
LD	Internal deletion	**2-mer**	**-**	**ND**

The second crystalline ortholog, Mb-sHSP 25, a larger version of this holdase chaperone encoded in the *M. burtonii* genome,was expressed and purified, and is stable at 65°C and able to stabilize boGDH at 42°C, however it is not as effective a salvage chaperone as the original homolog that we describe above. .

Publications in preparation (Year 3)

Luo, H, P. Laksanalamai and F. T. Robb. Determination of the role of the C-terminus in stabilization of the archaeal chaperonins. For submission to J. Molecular Biology.
Luo, H and F. T. Robb. Thermophilic protein folding systems. For submission to the Extremophiles Handbook, to be published in September 2010 (Springer, Tokyo).
Rowland, S, Luo, H and F. T. Robb. Protein salvage by combinations of chaperones from extremophiles. To be submitted to Biotechnology and Bioengineering)..